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The reaction of glutaraldehyde with proteins and other biological materials*

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SYNOPSIS

Aldehydes react primarily with the amino groups of proteins but under favourable conditions other groups may also be involved. Factors influencing the reaction such as time, temperature and pH are considered and the probable effects of interaction on the properties of the protein discussed.

With dialdehydes one or both aldehyde groups may react, leading in the first case to the introduction of an aldehydic group which may play a part in subsequent reactions and in the second to cross-linking of the polypeptide chains and increase in molecular size. The stability of the protein-aldehyde bonds varies with the aldehyde, those formed with glutaraldehyde being particularly stable.

Glutaraldehyde tends to polymerise, and at pH values above 8·0 evidence indicates that small oligomers are formed. These are soluble in a number of solvents including dioxan.

Brief mention is made of the possibilities of reaction of glutaraldehyde with other biological materials.

The fixation of biological specimens bears some analogies to tanning; in both cases one of the primary objectives is to stabilize the structure, reduce solubility and to make the material more resistant to enzymes and bacteria, while at a later stage the influence of the fixative or tanning agent on dye uptake is of importance. One big difference between the two processes however, is the range of conditions acceptable; for tanning these are relatively wide whereas with fixing these must be kept as nearly as possible in the physiological range.

In the main the contents of this paper are based on work carried out on the use of aldehydes as tanning agents. Thus, it is concerned mainly with the stabilization of proteins and in particular of collagen. Quantitatively results will differ from one protein to another but qualitatively they should follow the same general pattern and it is hoped that some of the information derived from these investigations will prove useful in the histochemical field.

Methods used to investigate interaction of aldehydes with collagen

Various methods have been used to gain information on the interaction of aldehydes with collagen.

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(1) Determination of aldehyde bound by collagen by chemical means (Bowes & Elliott, 1962) and by radio-isotope techniques using ^{14}C-labelled aldehydes (Bowes *et al.*, 1965).
(2) Determination of the number of amino groups involved in the binding by:
   (i) modification of the formol titration (Bowes & Cater—in preparation),
   (ii) amino acid analysis and determination of loss of lysine and hydroxylysine. This procedure will also indicate involvement of other amino acid residues.
(3) Measurement of the shrinkage temperature of the collagen. Native collagen shrinks sharply at 60–64°C and treatment with aldehydes raises this by 10 to 25°C.
(4) Estimation of the number of cross-links introduced using a physical method involving stress-strain measurements (Wiederhorn & Reardon, 1952; Cater, 1963). This method depends on the fact that after shrinkage collagen behaves like a rubber and equations developed for rubber-like polymers can be applied.

From a combination of these methods it is possible to arrive at certain conclusions regarding the mode of action of different aldehydes and to assess their potentialities as tanning agents and fixatives.

Interaction of aldehydes with proteins

The main groups in proteins which are likely to react with aldehydes are listed in Table 1. There is little evidence that the peptide bonds of the protein are involved in any way. The extent to which these various groups react is dependent on the conditions, particularly on the pH, and also varies from one aldehyde to another and to some extent with the protein configuration, some groups being apparently inaccessible, at least to the aldehydes of higher molecular weight.

Table 1. Reactive Groups of Proteins

Lysine	$-NH_2$
Arginine	$-NH.C(=NH)(NH_2)$
Histidine	$-CH_2-C(=CH-N)-CH-NH-$
Glutamine Asparagine	$-CONH_2$
Cysteine	$-SH$
Tyrosine	$-CH_2-C_6H_4-OH$
Tryptophan	$-CH_2-C_8H_6N$

Formaldehyde

Formaldehyde has been the aldehyde most frequently used for both tanning and the fixing of biological material. The behaviour of this simple aldehyde differs in a number of respects from that of the higher homologues and will, therefore, be

considered separately. Its interaction with proteins has been reviewed by French and Edsall (1945) Bowes (1948) and more particularly with collagen by Gustavson (1956).

Formaldehyde reacts readily with the amino groups and as the pH is raised to between 8 and 9 virtually all these groups become involved. With collagen it is this reaction which is primarily responsible for the stabilisation of the protein. Presumably it is also the reaction involved in fixing with formalin buffered in this pH range. Following the work of Fraenkel-Conrat and co-workers (1945; 1946; 1948; 1949) it is generally believed that the formaldehyde first reacts with amino groups and then condenses further with amide or guanidino groups

$$P.NH_2 + CH_2O \longrightarrow P.NH.CH_2OH$$

$$P.NH.CH_2OH + H_2N\text{–}C(=O).P' \longrightarrow P.NH.CH_2.NH.C(=O).P'.$$

amide

In the absence of amino groups, formaldehyde does not react with either amide or guanidino groups except under more extreme conditions of pH. At higher pH values of 10–12 the guanidino groups are directly involved but the interaction adds little to stability. Possibly a cyclic compound is formed involving both nitrogens of the guanidino group. The amide group can apparently react at low pH values under dehydrating conditions, for example, in the presence of salts, high concentrations of formaldehyde appear to be necessary to facilitate this reaction and as far as cross-links are concerned it appears to contribute little to stability.

Formaldehyde also reacts with sulphydryl groups in the neutral pH range, a cross-link being formed between two such groups (Middlebrook & Phillips, 1942; Brown & Harris, 1948). These groups are, however, often relatively inaccessible to chemical reagents. The imidazole group of histidine and the residues of tyrosine and trytophan also react with formaldehyde involving a Mannich-type reaction (i.e. condensation with an active hydrogen atom adjacent to a double bond and a secondary amine (see Fraenkel-Conrat & Olcott, 1948)).

As far as tanning is concerned such reactions are not generally considered to be of much importance since in collagen the amounts of the amino acids concerned are relatively low and the formation of cross-links is not involved. Histochemically they are likely to be of much greater importance since the specific staining reactions attributed to these amino acid residues will be affected. Certainly tyrosine can no longer be detected in acid hydrolysates of formaldehyde-treated collagen analysed by the Moore and Stein procedure (Moore *et al.*, 1958). The recovery of lysine, hydroxylysine, histidine and arginine is essentially complete so that although reaction with these does occur the bond with formaldehyde is broken during hydrolysis (Bowes & Raistrick, 1964).

Various other monoaldehydes have been examined as potential tanning agents but none have been found to be as satisfactory as formaldehyde.

Glutaraldehyde and other dialdehydes

Less information is available on the reaction of the dialdehydes with proteins and such work as has been done has been almost entirely confined to assessment of their tanning action. As with formaldehyde, stabilisation of the collagen is primarily associated with reaction of the amino groups. The shrinkage temperature of the

collagen is raised to 75–90°C depending on the aldehyde and the pH (Seligsberger & Sadlier, 1957; Fein & Filachione, 1957).

With glutaraldehyde reaction with amino groups begins to be appreciable at much lower pH values than with formaldehyde and at all such values considerably more is bound (fig. 1). In molar terms the difference is, of course, less (M.W. glutaraldehyde 100; formaldehyde 30) but the binding of glutaraldehyde is still greater by some 50 p.c. or more. The number of cross-links introduced is also considerably greater than with formaldehyde. Only amino groups appear to be involved in their formation and the number of cross-links introduced under optimum conditions of reaction approaches the maximum possible (Cater, 1963).

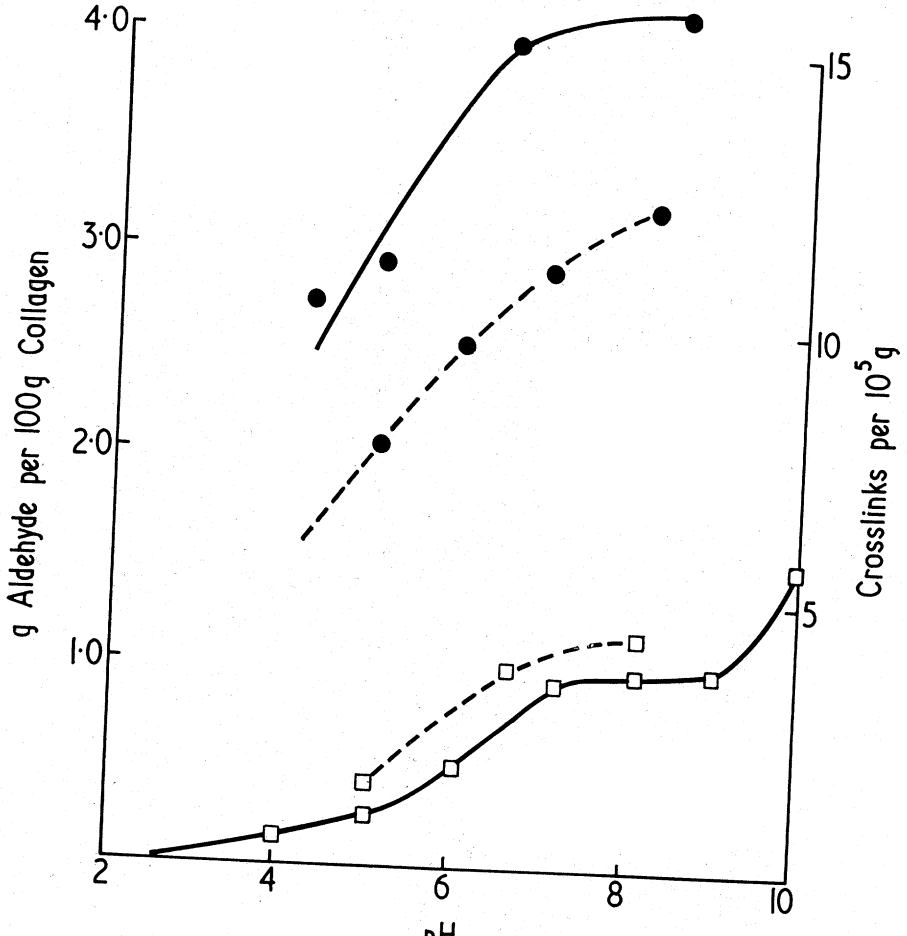

Fig. 1. Binding of Formaldehyde and Glutaraldehyde by Collagen.

●——● Glutaraldehyde bound

□——□ Formaldehyde bound (taken from Bowes and Kenten, 1949.

●- - - -● Cross-links introduced by glutaraldehyde

□- - - -□ Cross-links introduced by formaldehyde

Out of 34 moles amino groups per 10^5g collagen, approximately 26 moles appear to be available for the binding of glutaraldehyde, 20–22 of these under favourable circumstances are involved in cross-linking, leaving 4–6 moles involved in unipoint

fixation of the aldehyde (Bowes *et al.*, 1965). The molecular size of the glutaraldehyde molecule seems to be particularly suitable for bridging the gap between the amino groups of the polypeptide chains of collagen.

There is little evidence that the guanidino groups of arginine react with glutaraldehyde at pH values up to 9 or 10. Tyrosine is still found in acid hydrolysates of the tanned collagen, though sometimes in reduced amounts and it seems likely that reaction with this amino acid residue and also with tryptophan is less than with formaldehyde. No evidence is available regarding reaction with sulphydryl groups but there is no obvious reason why this should not occur.

Other dialdehydes, such as glyoxal, malonaldehyde, succinaldehyde and adipaldehyde appear to be less reactive than glutaraldehyde and fewer cross-links are formed. This may be an effect related to their molecular size or may be associated with differing solubility and stability. Such evidence as is available indicates that their reactivity to tyrosine is similar to that of glutaraldehyde. After treatment with glyoxal at pH 8·0 there is a large reduction in the amount of arginine which can be recovered from acid hydrolysates so that reaction with guanindino groups occurs even at this pH value. In contrast, formaldehyde, glutaraldehyde and malonaldehyde cause no significant decrease in arginine; the other aldehydes were not examined for this reaction (Bowes & Cater, in preparation).

Glutaraldehyde not only introduces more cross-links into collagen than other aldehydes but these are more stable. Boiling water and treatment in acid solutions has relatively little effect on the glutaraldehyde cross-links but causes appreciable reduction in those introduced by the other aldehydes (fig. 2). The relative efficiency of acrolein as a cross-linking agent suggests that it may be worth attention as a fixing agent especially where fixing in the vapour phase is indicated.

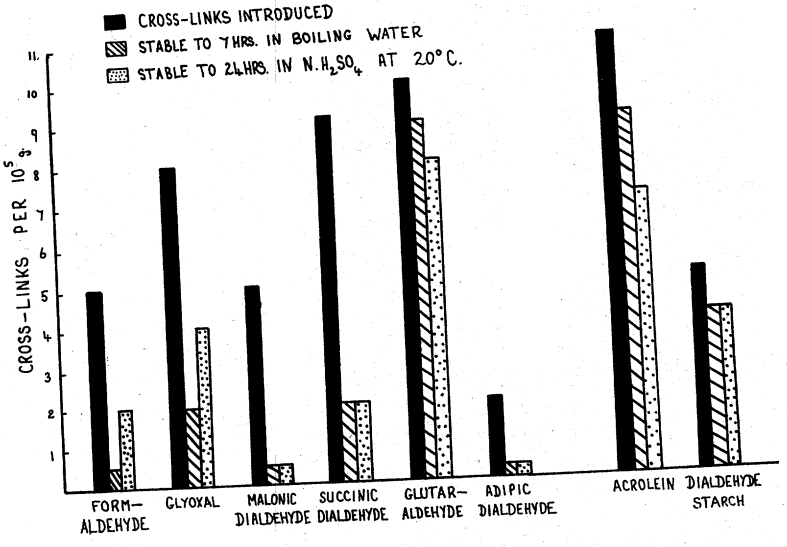

Fig. 2. Cross-linking with aldehydes. Moles of cross-links introduced per 10^5g and their stability. Reproduced from Bowes and Cater, 1965.

Little information is available regarding the action of glutaraldehyde on tissues other than protein. Presumably it will react with substances containing amino and probably sulphydryl groups but not one would think with mucopolysaccharides.

As expected it reacts with amino acids and small peptides; for example, when it is added to a solution of glycine there is an immediate darkening in colour and the inference is that it causes polymerization by linking the glycine molecules through the amino groups. No precipitate was formed, however.

Properties of glutaraldehyde and factors influencing its reaction with proteins

Glutaraldehyde tends to polymerize readily and is difficult to obtain in the pure state as a monomer, small amounts of water catalysing the polymerization. It is, therefore, generally supplied as a 25 p.c. solution having the slightly acid pH of about 4 to 5. In this it exists as a hydrate and experience in these laboratories suggest that such solutions are relatively stable for long periods of time. There is some darkening of the solution but no evidence of any extensive polymerization or loss of aldehyde groups. As the pH is raised, however, polymerization increases and above pH 9·0 there is quite extensive loss of aldehyde groups (Fein *et al.*, 1959). Recently papers have been published by two Japanese workers (Aso & Aito, 1962) indicating that small oligomers are formed which on average contain three glutaraldehyde residues and have one free aldehydic group. In tanning experiments the amounts of glutaraldehyde bound exceed a one to one ratio with the amino groups. Allowing for two amino groups being involved in each cross-link, this suggests that on average two or three molecules of glutaraldehyde are linked together in each cross-link and at each centre of unipoint fixation. Aso and Aito report, however, that the oligomers are soluble in dioxan, chloroform, tetrahydrofuran and dimethyl formamide, and extraction of tanned collagen with the first of these solvents led to the removal of appreciable amounts of material giving aldehydic reactions.

It seems probable, therefore, that some of the glutaraldehyde in the tanned collagen is merely deposited as a polymeric material and that the oligomers are not necessarily bound by the collagen.

Reaction with glutaraldehyde is favoured by rise in pH, temperature and concentration of aldehyde, the first probably being the most important. Some reaction occurs even at pH 3·0, but the amounts bound increase sharply as the pH is raised. The optimum pH is in the neighbourhood of 8·0, at higher pH values the tendency of the aldehyde to polymerize reducing the efficiency as a cross-linking agent. Under favourable conditions reaction is relatively rapid and diffusion is probably the main rate-determining factor. In tanning experiments it is generally found to be convenient to impregnate the skin under conditions where the rate of reaction is slow e.g. at pH 3 to 4 and then to raise the pH and temperature at a later stage. Presumably for fixing biological material the use of low pH values may not be acceptable and in such cases the temperature should be kept as low as possible. In order to reduce the incidence of artifacts due to polymerization it is probably advisable not to exceed pH 7·0 and certainly not 8·0.

In view of its tendency to polymerize in the presence of very small amounts of water it seems doubtful whether glutaraldehyde can be used in the vapour phase.

DISCUSSION

From the data presented it is clear that in the main it is reaction of aldehydes with the amino groups of the protein which is responsible for their stabilizing action in tanning and in fixing. Cross-links between the polypeptide chains are formed, the molecular weight is thereby increased and the solubility decreased and the molecular configuration retained.

There are also other effects on the protein which are of relevance to histochemical techniques. The charge on the protein will be affected, substitution of the amino

groups leading to a reduction in the positive charge on the protein. In consequence this will carry a net negative charge over a wider pH range and the uptake of acid dyes will, therefore, be reduced, and that of basic dyes will be increased. At low pH values, where the carboxyl groups are mainly uncharged, the uptake of acid dyes should not be greatly affected, except in so far as the increased stability of the molecular structure retards penetration. There will also be changes in specific dyeing properties depending on the conditions of the reaction and the nature of the aldehyde. The uptake of reactive dyestuffs such as those of the triazine type is reduced by the blocking of the amino groups with glutaraldehyde. Staining reactions involving imidazole, tyrosine or tryptophan residues may also be affected, more particularly by formaldehyde. Staining for electron microscopy using such reagents as phosphotungstic acid or other compounds reacting with lysine and arginine residues will also be reduced by most aldehydes, perhaps more specifically by glyoxal which appears to react with both these residues.

Finally the possibility of artifacts arising from deposition of polymeric material or the introduction of aldehyde groups must be borne in mind. The first can be reduced by limiting the upper pH limit used in fixing and by thorough washing. With glutaraldehyde an extraction with dioxan to remove the oligomers reported to be formed may be a useful precaution. The possibility of free aldehyde groups remaining and interfering with staining reactions is of particular importance with the dialdehydes. Evidence suggests that with glutaraldehyde up to 4 moles per 10^5g protein may be unipointly fixed by amino groups. Sections of skin fixed with 5 p.c. glutaraldehyde at pH 6·0 gave an intensive colour reaction with Schiffs reagent without preliminary oxidation by periodic acid indicating the presence of such free aldehyde groups. The reaction was more intense than that obtained by similar formol-fixed tissue. While glutaraldehyde appears to be by far the most efficient aldehyde for tanning and fixing in solution, it seems doubtful whether it will be useful as a vapour fixative, firstly because of its low volatility and secondly because of its tendency to polymerise unless all traces of water are removed.

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